APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2002, p. 397–400 Vol. 68, No. 1
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.1.397–400.2002

Transmission of *Escherichia coli* O157:H7 from Contaminated Manure and Irrigation Water to Lettuce Plant Tissue and Its Subsequent Internalization

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The transmission of *Escherichia coli* O157:H7 from manure-contaminated soil and irrigation water to lettuce plants was demonstrated using laser scanning confocal microscopy, epifluorescence microscopy, and recovery of viable cells from the inner tissues of plants. *E. coli* O157:H7 migrated to internal locations in plant tissue and was thus protected from the action of sanitizing agents by virtue of its inaccessibility. Experiments demonstrate that *E. coli* O157:H7 can enter the lettuce plant through the root system and migrate throughout the edible portion of the plant.

In recent years, *Escherichia coli* O157:H7 has been isolated with increasing frequency from fresh produce, including bean sprouts, cantaloupes, apples, and leaf lettuce (1, 10). The mechanisms by which the pathogen is introduced into the lettuce plant are not fully understood; however, one hypothesis states that the plant becomes contaminated when grown in fields fertilized with improperly treated manure (3). Epidemiological data indicate that *E. coli* O157:H7 may be present in up to 8.3% of dairy and beef cattle (8) and that it is shed asymptomatically in the feces. Current manure-handling guidelines suggest a composting period before application of the manure to a field as fertilizer (9). Research has demonstrated the long-term survival of *E. coli* O157:H7 in manure held under a variety of conditions (11, 15), so even strict adherence to the guideline may result in the application of manure containing culturable *E. coli* O157:H7 to production fields (15).

A second vehicle by which *E. coli* O157:H7 may be introduced is flood irrigation with water contaminated with cattle feces or contact with contaminated surface runoff (1, 10). A number of recent *E. coli* O157:H7 outbreaks have been linked to contaminated water (6); furthermore, studies have demonstrated the ability of the pathogen to survive for extended periods in water (7, 16). Cattle in an adjacent field were implicated as the source of *E. coli* O157:H7 during a multistate outbreak associated with the consumption of mesclun lettuce in 1996 (10). The authors speculated that contaminated water was used to irrigate the lettuce fields.

Lettuce production practices commonly include a rinse step in which the leaves are sanitized using tap water containing 100 to 200 ppm of free chlorine (2). This level of chlorine has been shown to be only marginally effective at reducing the level of *E. coli* O157:H7 on lettuce tissue surfaces (3). The ineffectiveness of chlorine and other surface-sanitizing agents is likely dependent on whether the target organisms are readily accessible. Cells of *E. coli* O157:H7 were shown to penetrate into the stomata and junction zones of cut lettuce leaves, becoming entrapped 20 to 100 μm below the surface of the cut edge (12). Cells entrapped at subsurface locations were protected from sanitation with chlorine.

Previous studies have not provided a direct link for contamination of lettuce in the field through fertilization with *E. coli* O157:H7-contaminated manure or irrigation with contaminated water. Moreover, the sites of association, surface or subsurface, of the pathogen following in-field contamination have not been delineated. We investigated whether *E. coli* O157:H7 associated with contaminated manure or irrigation water can be transported from the root system into the edible portion, putatively by the plant vascular system. In this study, we demonstrated the transmission of *E. coli* O157:H7 to lettuce plants from contaminated manure incorporated into the soil. Furthermore, the contamination of lettuce through flood irrigation with contaminated water was demonstrated. *E. coli* O157:H7 expressing green fluorescent protein (GFP) was used to facilitate detection of the target organism in association with lettuce tissue.

Bacteria. *E. coli* O157:H7 (ATCC 43895) was transformed using the pGFP plasmid (Clontech, Palo Alto, Calif.), encoding GFP. The GFP reporter system was selected for its utility in visualizing bacteria in biological systems and because cells can be studied nondestructively, without further processing or substrate addition (4). GFP-expressing *E. coli* O157:H7 (*E. coli*

TABLE 1. Detection of *E. coli* O157:H7 associated with lettuce seedlings treated with $HgCl_2$

Surface examined	Sample day	No. of positive samples according to soil concentration of *E. coli* O157:H7[a]					
		10^4 CFU g^{-1} soil		10^6 CFU g^{-1} soil		10^8 CFU g^{-1} soil	
		5 min	10 min	5 min	10 min	5 min	10 min
Inner	3	0/8	0/8	0/8	0/8	6/8	2/8
	6	0/8	0/8	2/8	1/8	3/8	2/8
	9	2/6	0/6	1/6	3/6	3/6	2/6
Outer	3	0/8	0/8	2/8	0/8	2/8	2/8
	6	0/8	0/8	1/8	0/8	0/8	0/8
	9	0/6	0/6	0/6	0/6	0/6	0/6

[a] Number of *E. coli* O157:H7-positive sections/total number of sections. Five minutes and 10 min refer to treatment times of sprouts in 0.1% $HgCl_2$.

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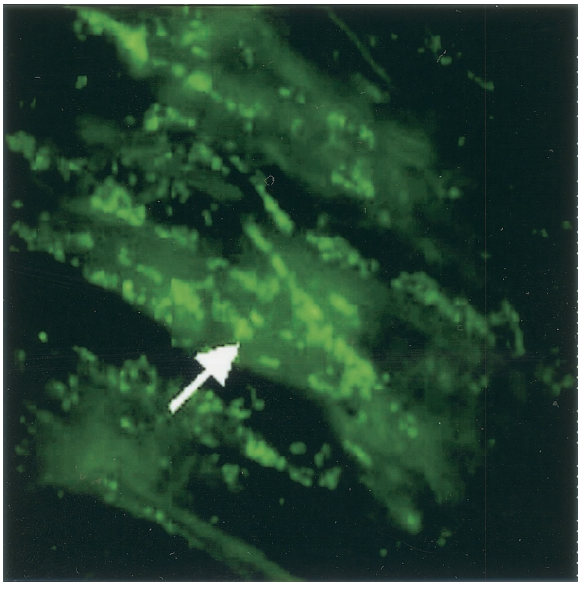

FIG. 1. Photomicrograph showing colonization of the surface of a 3-day-old lettuce seedling grown in soil containing 10^6 CFU of *E. coli* O157:H7/pGFP g^{-1}. Cells appear as aggregates and attach preferentially to junction zones of lettuce cells. The arrow indicates foci of *E. coli* O157:H7 cells.

O157:H7/pGFP) was cultured at 37°C for 24 h in tryptic soy broth (Difco, Cockeysville, Md.) supplemented with 100 μg of ampicillin (Sigma, St. Louis, Mo.) ml^{-1}. The cells were harvested by centrifugation (3,500 × *g*; 10 min.) and resuspended in sterile distilled water (SDW). Inocula were prepared by serial dilution in SDW to achieve the desired cell concentrations. All experiments were conducted using *E. coli* O157:H7/pGFP.

Preparation of planting mixture. Fresh cow manure (475 g) collected at the Rutgers University dairy barn was inoculated with a suspension of *E. coli* O157:H7/pGFP and vigorously mixed by hand. Manure collected from the farm for inclusion in research experiments is routinely screened for the presence of *E. coli* O157:H7 and is consistently negative. The manure was collected immediately following evacuation from the animal and was used in experiments within 48 h. The inoculated manure was then mixed with 4.5 kg of soil (sandy loam; pH 7.13) to give 5 kg of planting mixtures with final *E. coli* O157:H7/pGFP concentrations of approximately 10^8, 10^6, and 10^4 CFU g^{-1}. The planting mixtures were dispensed into vegetable flats, and seeds of green ice lettuce (lot no. 52977; W. Atlee Burpee & Co., Warminster, Pa.) were planted. The flats were kept at 20°C, illuminated for 14 h using Agro-Lite lights (Philips Lighting Company, Somerset, N.J.), and watered daily.

Sampling procedures and detection by culture methods. On days 3, 6, and 9 postplanting, seedlings were collected from each flat. The seedlings were cut from the root systems approximately 1 cm above the soil surface to minimize surface contamination of the edible portion of the plant through contact with the planting mixture. The seedlings were surface disinfected by being dipped in 80% ethanol for 5 s followed by immersion in 0.1% (wt/vol) $HgCl_2$ for either 5 or 10 min. The seedlings were washed twice in sterile water and allowed to air

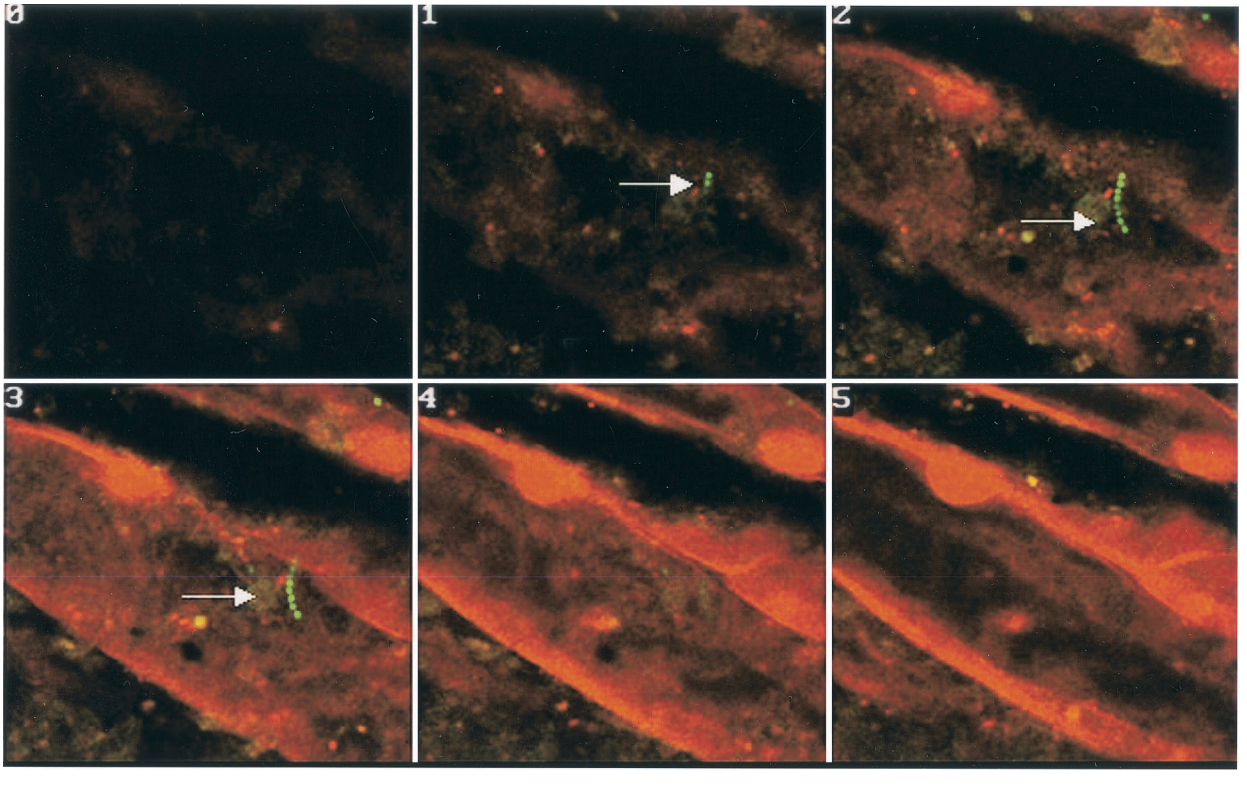

FIG. 2. Representative LSCM optical thin section of a lettuce seedling contaminated with *E. coli* O157:H7/pGFP. *E. coli* O157:H7/pGFP cells can be found in the subsurface tissue of the seedling. *E. coli* O157:H7/pGFP cells appear green (arrows), while lettuce tissue appears red. Each successive image progresses 1 μm deeper into the lettuce seedling.

dry at room temperature in a laminar flow hood. Of the 16 seedlings treated for 5 min, 8 were placed directly on tryptic soy agar (TSA) plates supplemented with 100 μg of ampicillin (Amp) ml^{-1}. The remaining eight seedlings were sliced longitudinally to the base of the cotyledons, and the inner surfaces were placed on TSA-Amp plates. After incubation at 37°C for 1 h, the seedlings or sections of seedlings were removed and the plates were further incubated at 37°C overnight. The 16 seedlings immersed in $HgCl_2$ for 10 min were examined as described above. The plates were illuminated with UV light, and GFP-expressing colonies were enumerated. *E. coli* O157:H7/pGFP was recovered from the surfaces of sanitized seedlings grown in planting mixtures containing the highest levels of the target pathogen (Table 1). Based on culture, 10-min exposure of exterior surfaces of seedlings to $HgCl_2$ eliminated most culturable bacteria, suggesting that the target pathogen was located within the seedling tissue and therefore was protected from the action of the sanitizing agent. Under the experimental conditions outlined in the present study, *E. coli* O157:H7 maintained the plasmid encoding GFP.

Fluorescence microscopy and laser scanning confocal microscopy (LSCM). Sections of seedlings were further examined by fluorescence microscopy on days 3, 6, and 9 postplanting. Samples were stained with propidium iodide (10 μg ml^{-1}; Molecular Probes, Eugene, Oreg.) for 30 min, washed twice in phosphate-buffered saline (Sigma), and then mounted on glass microscope slides and examined with an Olympus BH-2 epifluorescence microscope equipped with a 100× oil objective. Images were captured with a charge-coupled device camera (Photometrics, Tucson, Ariz.) and formatted using Adobe Photoshop. Cells of *E. coli* O157:H7/pGFP were visualized on the cotyledons and hypocotyl of the lettuce seedlings, regardless of the level of soil contamination or day of sampling (Fig. 1). The surfaces of the seedlings likely became contaminated as the seedlings grew and broke through the soil surface.

Based on fluorescence microscopy, seedlings found to contain surface-associated *E. coli* O157:H7/pGFP were further examined using LSCM to determine if the target pathogen was located below the tissue surface. Slides were examined using a Zeiss Axioplan 410 microscope equipped with an Ar-Kr laser source and a 100× oil objective. *E. coli* O157:H7/pGFP was excited using the 488-nm laser line. Propidium iodide-stained tissue was excited with the 568-nm laser line. Emissions were detected using a 515- to 540-nm band-pass filter for *E. coli* O157:H7/pGFP and a 590-nm long-pass filter for propidium iodide-stained lettuce tissue. Confocal images were captured and merged using the Zeiss LSM software. In some instances, target bacteria were not visualized on the surface of lettuce tissue but were found in high numbers at subsurface locations (Fig. 2). The target pathogen was visualized at depths of up to 45 μm below the tissue surface, suggesting migration to an internal location (Fig. 3). These results confirm the culture results of the present study (Table 1) and previous studies (13) indicating *E. coli* O157:H7 can localize within lettuce tissue.

Effect of irrigation with contaminated water and manure slurry. To determine whether direct surface contact with the edible portion of the plant is required for internal contamination, 25 green ice lettuce plants were grown in 15-cm-diameter plastic pots containing Pro-Mix BX (Premier Horticulture Inc., Red Hill, Pa.). The plants were fertilized weekly with Peter's

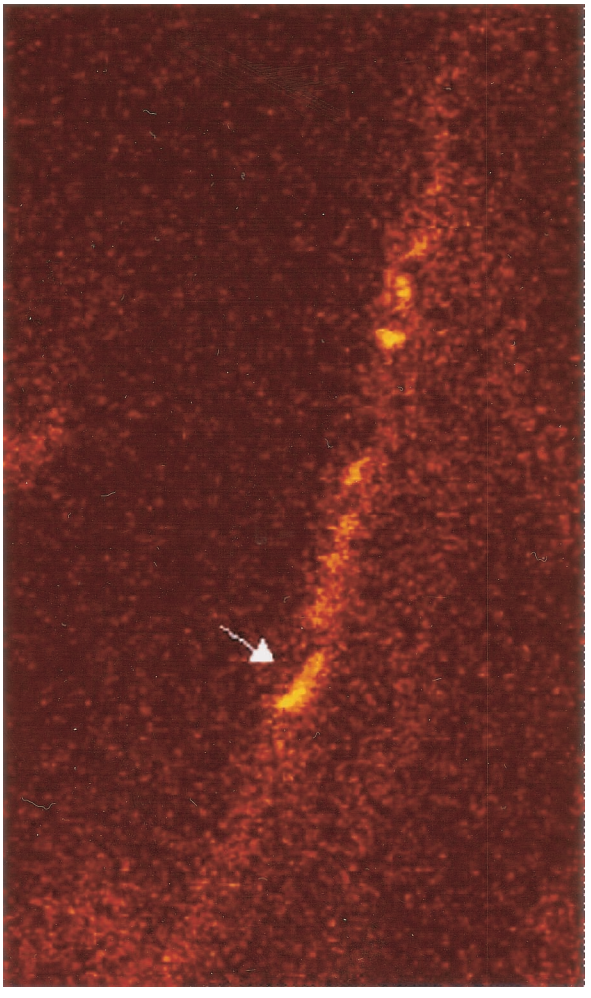

FIG. 3. LSCM photomicrograph of lettuce leaf showing cells of *E. coli* O157:H7/pGFP at an internal location 45 μm from the outer leaf surface. *E. coli* O157:H7/pGFP cells (arrow) were not randomly dispersed but rather formed a band of aggregates restricted to the intercellular space.

General Purpose 20-20-20 fertilizer (Grace Sierra Horticultural Products, Milpitas, Calif.) in the Rutgers University greenhouse. Mature plants (approximately 50 days old) were moved to our laboratory and bundled with twine to prevent the edible portion of the plant from touching the soil. *E. coli* O157:H7/pGFP was processed as described above and resuspended in SDW. The soil in each of 15 pots was irrigated with 200 ml of water containing 7.5×10^7 CFU of *E. coli* O157:H7/pGFP ml^{-1}. The inoculum was applied carefully to prevent splashing of the inoculum onto the edible portion of the lettuce plant. Five plants were harvested on days 1, 3, and 5 postinoculation and processed as follows. The plants were cut 2 cm above the soil surface with a sterile scalpel; the entire edible portion of the plant was combined with 200 ml of SDW in a sterile polyethylene bag and homogenized for 2 min in a stomacher (Dynatech Laboratories, Alexandria, Va.). The liquid phase was removed, centrifuged (3,500 × *g*; 10 min.), resuspended in 1 ml of SDW, and plated onto the surface of a

TABLE 2. Detection of *E. coli* O157:H7 in edible lettuce tissue following plant growth in soil exposed to contaminated irrigation water or manure slurry

Day post-exposure	Soil exposure[a]	
	Contaminated irrigation water	Contaminated manure slurry
1	4/5	4/5
3	2/5	3/5
5	2/5	ND

[a] Number of plants positive for *E. coli* O157:H7/number of plants tested. ND, no plants were tested.

TSA-Amp plate. The plates were incubated at 37°C overnight, and GFP-expressing colonies were visualized under UV light.

Contamination of the edible portion of the lettuce plant through exposure of soil, and consequently the plant root system, to manure runoff was also examined. Manure slurry was prepared by the method of Calicioglu et al. (5) and inoculated to achieve a concentration of 1.25×10^8 CFU of *E. coli* O157:H7/pGFP ml^{-1}. Inoculated slurry (200 ml) was applied to the soil of the 10 remaining lettuce plants. On days 1 and 3 postinoculation, five plants were processed as described above, and the presence of *E. coli* O157:H7/pGFP colonies was determined. The results indicate that *E. coli* O157:H7 is capable of entering the roots of mature lettuce plants and can be transported upward to locations within the edible portions of the plant (Table 2). Direct contact between the leaves and a contamination source is not required for the organism to become integrated into edible lettuce tissue.

Application of *E. coli* O157:H7-contaminated manure to the production field or irrigation with *E. coli* O157:H7-contaminated water may result in contamination of the crop in the field. Studies have indicated that *E. coli* can survive for extended periods in manure and water (7, 11). We have demonstrated that lettuce grown in soil containing contaminated manure or irrigated with contaminated water results in contamination of the edible portion of the lettuce plant. Moreover, the results suggest that edible portions of a plant can become contaminated without direct exposure to a pathogen but rather through transport of the pathogen into the plant by the root system. We recognize that the levels of *E. coli* O157:H7 used in this study are far greater than what may be found on an agricultural field; however, numbers of bacteria were used that could be readily detected by the assays used in the present study. Under natural conditions, even a low level of contamination could present a significant human health risk, since the infective dose of *E. coli* O157:H7 is less than 1,000 cells (1). Research suggests that surface sanitizing of lettuce is not an effect method to eliminate all *E. coli* O157:H7 cells (3, 14). The inaccessibility of a large number of organisms, as a consequence of their subsurface location, is perhaps the reason for the lack of effectiveness of surface-sanitizing treatments. The impacts of on-farm practices which may result in *E. coli* O157:H7 becoming associated with lettuce, or for that matter other crops, have not been sufficiently explored.

This work was supported by a USDA grant (99-04872), and E.B.S. is supported by a USDA National Needs Fellowship.

We thank Joe Florentine of the NJAES Research Greenhouse for assistance with plant cultivation and John Bugowski of the Rutgers University Dairy Farm.

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